

THE BIOCHEMISTRY OF HUMUS FORMATION AND SOME PROBLEMS OF PLANT NUTRITION

by

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The biochemistry of humus formation is one of the most complicated aspects of the soil-humus problem and its study is of great theoretical and practical importance.

Various theories have been put forward on the role of different components of vegetable and animal origin in the formation of humic substances and on the mechanism of this process. For a long time the view was widely held that easily mobile constituents of organic residues cannot participate in humus formation, because micro-organisms decompose them with formation of completely mineralized end-products and low-molecular organic acids. According to this view the participation of the carbohydrates (simple carbohydrates, cellulose, hemicelluloses) in the formation of humic substances was excluded although they represent about 50% of the total mass of plant residues. The main source of humic substances was considered to be the more resistant components of plant residues, particularly lignin, which are converted into humic substances by oxidation and condensation.

These ideas, first put forward by German coal chemists (Fischer, Schrader, Fuchs, *et al*) during 1920-1930, were extended into the field of soil-humus study by Waksman in the 1930's. Assuming that humic substances contain nitrogen, Waksman considered that they were formed as a result of the condensation of lignin with protein, the latter being considered to be a product of microbial resynthesis.

Without going into a critical examination of Waksman's concept of the ligno-protein concept (this has already been done by Kononova, 1951), we shall show that this concept was reflected later in the theories of the Swedish scientists Mattson and Koutler-Andersson (1942, 1943) (see Kononova, 1953). According to these theories also, lignin is regarded as the main source of humic substances, but is subjected during humification to oxidation and to condensation, with ammonia liberated during the decomposition of nitrogen-containing components of plant residues; furthermore, nitrogen enters the aromatic rings of lignin and forms complex cyclic compounds characteristic of humic substances.

It should be noted that this concept limited not only the possibility of various components of plant tissues participating in humus formation, but also the role of micro-organisms in this process. Micro-organisms were regarded only as agents in the decomposition of plant residues; the condensation of lignin with protein (or with NH_3) was regarded as a physico-chemical reaction occurring without participation of micro-organisms.

According to contemporary views, formation of humic substances is a complex two-phase process including (1) decomposition of original plant and animal residues to simpler compounds and (2) subsequent synthesis of particles of specific high-molecular organic substances, which appear to be humic substances.

At the present time the principle of the structure of particles of humic substances can be regarded as fairly well known. In spite of variations in different soils, peats and coals, humic substances (including humic and fulvic acids) are characterized by a series of common characteristics. Carbonized substances are representatives of these groups; the presence of an aromatic ring in the form of a flat atomic lattice of carbon linked with side-chains including various functional groups (among them carboxyl) is characteristic. The presence of nitrogen both in cyclic forms and in the side-chains is also characteristic of soil humic substances.

A study of the biochemistry of the formation of humic substances in conjunction with a study of the structure of their particles permits us to assume that condensation of an aromatic compound of the polyphenol type with a nitrogenous compound of the amino-acid or peptide type is the basis of the formation of the primary particle of humic substances. It should be noted that similar reactions are widespread in the plant and animal world, particularly during pigment formation.

The question of the sources of humic substances is also fairly clear now. Contrary to previous ideas, in which the origin of humic acids was connected with particular plant substances (lignin, carbohydrates, proteins) the problem is considered

at the present time from the point of view of the origin of components for the formation of the primary particles of humic substances (i.e., aromatic compounds of polyphenol, amino-acid or peptide type). It has been shown that various plant substances, subjected during humification to complicated biochemical transformations, can serve as sources of these components.

Thus "building units", liberated during the decomposition of lignin, and tannic substances of aromatic structure can serve as sources of the aromatic ring. Plant respiratory enzymes which have an aromatic structure, or certain products of microbial metabolism, in which various plant substances including carbohydrates are utilized as the primary source of energy, can also be a source of the aromatic ring.

As regards the second component for building the primary particle of humic substances—amino acids and peptides—there is reason for assuming that they represent the products of microbial resynthesis, because the nitrogen-containing components of the original plant residues are utilized fairly rapidly by micro-organisms.

So far as the aromatic nature of humic substances is concerned, the possibility of the formation of similar substances and of melanoid-type substances, by means of condensation of compounds with an open chain (e.g., uronic acids) with peptides or amino acids, should be borne in mind. However, the nature and properties of such types of humic substances require investigation in detail: dark colour and participation in the formation of a water-stable soil structure are not sufficient for their characterization.

An important feature of the biochemistry of humus formation appears to be the fact that the second condensation phase of the process, during which the primary particles of humic substances are formed, proceeds under conditions of biocatalysis accomplished by oxidizing enzymes of the phenoloxidase type, produced by numerous micro-organisms.

As a result of the effect of these enzymes the oxidation of polyphenols to quinones is accelerated; the latter are subsequently condensed with peptides or amino acids.

We present below the results of investigations which illustrate the diversity of ways by which humic substances are formed and the complicated and sometimes unexpected transformations to which the original substances are subjected during the process.

Experimental

Investigations were carried out on pure cultures of the mould fungi *Aspergillus niger* and *Penicillium* spp. which, as had been shown beforehand, form dark-coloured products during growth.

Experiments were carried out in conical flasks of half-litre capacity, each containing 300 ml liquid nutrient medium consisting of mineral salts KH_2PO_4 , KCl , MgSO_4 , FeSO_4 , ZnSO_4 and with glucose as the sole source of energy; nitrogen was added to the medium as NaNO_3 .

As fungal growth progressed the colour of the nutrient solution gradually became dark brown or cherry-reddish; this colouration was obviously connected with the advancing autolysis of the fungal hyphae and the formation of humus substances.

Flasks were periodically removed one by one from the experiment. After the culture liquid had been separated from the hyphae by filtering, the pH, the amount of residual sugar (using Bertrand's method) and the amount of protein nitrogen (precipitation according to Barnstein) were determined; qualitative tests for the presence of substances of aromatic nature were also carried out.

Stationary observations on changes in the oxidation-reduction potential Eh of the culture liquid were also made in flasks (with platinum electrodes and agar bridges) specially constructed for each series. The Eh was measured by the calomel electrode.

All determinations were carried out on the 3rd, 6th, 9th, 14th, 20th and 29th days from the beginning of the experiment, and subsequently every 10 days.

The results of the pH determination in the medium are given in Table 1.

Table 1

Changes in the pH values of the medium during experiments

Culture	pH values at different times (days)							
	0	3	9	20	29	39	49	60
<i>Aspergillus</i>	4.20	—	4.45	4.64	5.36	5.79	5.59	—
<i>Penicillium</i>	4.20	4.58	5.05	5.50	6.18	6.37	6.52	6.5

Table 1 shows that with *Penicillium* a shift towards neutrality occurred in the first days of development of fungal hyphae; after one month the pH value reached 6.18 and subsequently continued to increase slightly. In the experiment with *Aspergillus* the shift in the reaction was markedly slower and even after 50 days the medium still remained acid.

By comparing the data on changes of pH of the culture liquid with the curves illustrating the utilization of sugar and the appearance in the medium of protein nitrogen (Fig. 1) it can be concluded that the sharpest shift of the pH towards neutrality in both cultures coincides with the time at which the fungal requirement for sugar is maximal and with the beginning of autolysis of the fungal hyphae.

The results of determinations of the oxidation-reduction potential Eh in experiments with cultures of *Aspergillus* and *Penicillium* are illustrated graphi-

cally in Fig. 2. In both cases the Eh increased gradually, the transition from negative to positive values occurring approximately on the 25th to 29th day of the experiment; this also coincided with the period of maximum utilization of the initial organic substance, glucose, and with the accumulation in the medium of products of autolysis.

Further autolysis of the fungal hyphae and formation of new humic substances proceeded at positive values of the oxidation-reduction potential. Calculations of rH_2^* show that formation of new humic substances proceeded in our experiments under aerobic conditions: the rH_2 values in both series were 23-25.

The appearance of aromatic substances in the culture liquid is of great interest. In the culture of *Penicillium* glucosides were detected (reaction with

resorcin and H_2SO_4) on the third day, and compounds of pyrogallol and aromatic-acid types (reaction with $FeCl_3$) on the ninth day.

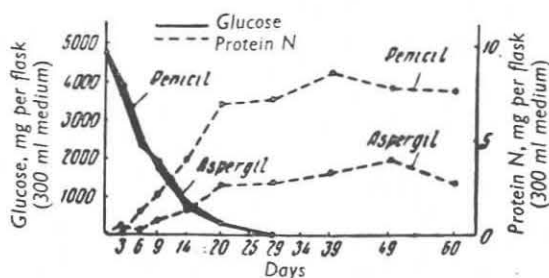


Fig. 1. Amounts of glucose and protein nitrogen in cultures of mould fungi

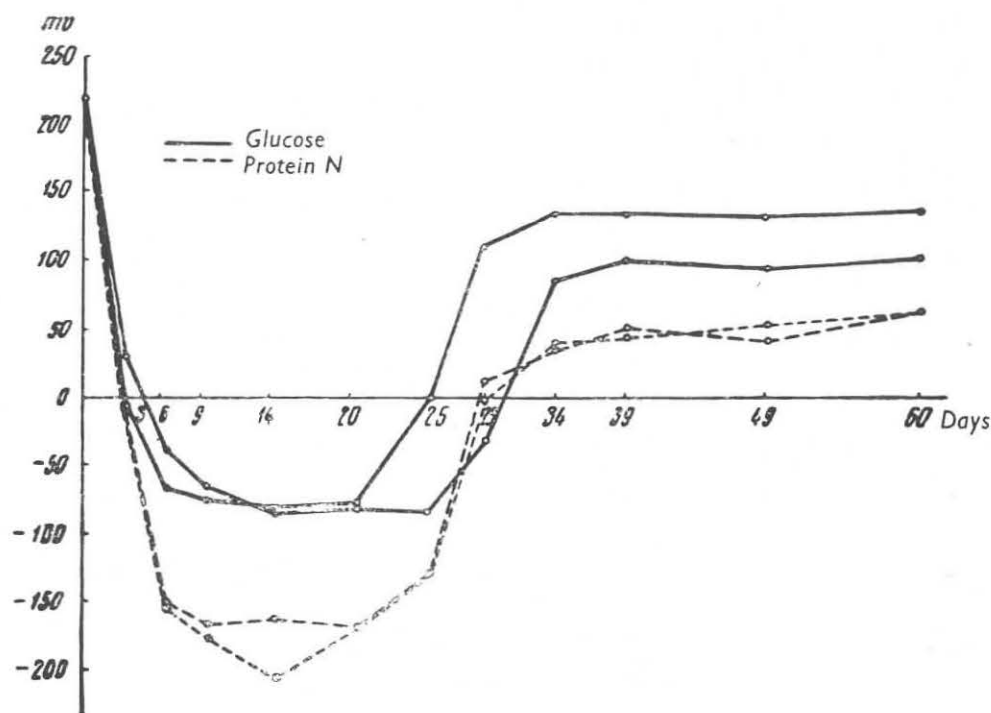


Fig. 2. Eh values in experiments with *Aspergillus* and *Penicillium*

* In calculating rH_2 , the Eh values were corrected for the hydrogen electrode, i.e., increased by 250 mv

$$rH_2 = \frac{Eh (v)}{0.029} + 2 pH$$

It should be noted that in the *Aspergillus* culture liquid the presence of aromatic compounds was detected much later and not so clearly.

Aromatic compounds are apparently the products of microbial metabolism; the fact of their appearance in a medium not containing initially any other organic compounds except carbohydrate (glucose) confirms the possibility of the biochemical conversion of mobile compounds with an open chain into resistant, cyclic compounds which subsequently serve as constituents for the synthesis of primary particles of humic substances.

No less interesting is the presence in the culture of protein nitrogen, which appears to be a product of the activity of fungi converting the mineral nitrogen of the medium into organic forms.

Qualitative tests (experiments with oxidation of pyrogallol with and without addition of H_2O_2) showed the presence in both cases of enzymes of the phenoxidase type, that apparently produce conditions of biocatalysis during the condensation of aromatic compounds with organic forms of nitrogen.

Furthermore, our aim was to study the nature of newly formed humic substances. For this purpose an experiment with a series of flasks containing a medium of the above-mentioned composition was carried out. After the experiment had been completed, the dark-coloured culture liquid separated from the hyphae was evaporated on a water bath at neutral reaction and at a temperature not higher than $50^\circ C$; the liquid was freed from mineral compounds and water-soluble organic substances by dialysis (firstly by water-dialysis in small cellophane bags, then by electro-dialysis) and, after being made completely clear by repeated filtering, the coagulated liquid was air-dried, after being poured into glass dishes, so as to form a thin layer.

In the preparations obtained the elementary composition (micro-method) and absorption capacity (potentiometric titration; Khainskil, 1936) were determined. The forms of nitrogen made soluble during the hydrolysis of the preparations by 2% and 6 n HCl were determined by partition paper

chromatography; special investigations using infra-red spectroscopy permitted the isolation of certain groups characterizing the aromatic structure of the substances studied.

The most important results of the investigations are shown below.

Data on the elementary composition and exchange capacity are given in Table 2. For purposes of comparison figures characterizing the elementary composition and exchange capacity of humic acids and fulvic acids of podzolic soil are given in the last two lines of the table.

These investigations show that newly formed humic substances appear to be complex compounds of acid nature, capable of exchange reactions; the latter is shown by the similarity of the exchange capacity of newly formed humic substances with that of humic acids of podzolic soil. However, newly formed humic substances are characterized by a certain "formlessness" or low degree of condensation, shown, in particular, by the low values of the C/H ratios.

Of great interest are the results of the study of the forms of nitrogen in humic substances that we carried out using partition paper chromatography. Detailed results of these investigations were given in Kononova and Aleksandrova (1956); here we shall only point out that amino acids, detected both in the acid hydrolysates of these substances and in hydrolysates of humic substances of the soil, indicate the protein nature of the major part of the nitrogen of humic substances. In addition to this the amino-acid composition in the various materials was generally similar (Fig. 3).

Infra-red spectroscopy of newly formed humic substances and of soil humic and fulvic acids indicated the presence of the following groups, which confirm the aromatic structure of their particles: C-H (band at 3.3μ), C=C (doublet at $6.2-6.5\mu$) (Kasatochkin and Zil'berbrand, 1956).

Thus comparison of the results of investigations on the biochemistry of processes occurring in the

Table 2
Comparative characteristics of humic substances

Humic substances	% dry ash-free substance				C/H	pH	Exchange capacity (m e/100 g at pH 7)
	C	H	O	N			
In experiment with <i>Aspergillus</i> ...	51.0	5.6	40.6	2.8	9.1	3.42	312
" " " <i>Penicillium</i> ...	45.2	6.1	45.9	2.8	7.4	3.26	261
Humic acids from strongly podzolized soil	56.5	3.8	33.8	5.9	14.9	3.3	324
Fulvic acids from the same soil ...	49.9	3.3	44.9	1.9	15.1	2.7	651

fungal cultures with the data characterizing the nature of the humic substances formed enables the following conclusions to be drawn.

1. The humic substances formed in the cultures do not differ basically from soil humic substances. They are of aromatic nature and contain nitrogen, partially represented by protein forms; they possess functional groups, the hydrogen of which is capable of exchange reactions.

2. In as much as glucose was the sole source of organic matter in the initial culture liquid it is clear that, in our experiment, the products of fungal activity (products of resynthesis and metabolism) were "structural units" in the formation of the primary particles of humic substances.

We are far from thinking that the example we have given is the only way in which humic substances are formed in nature, but it does illustrate the complexity of these ways and the close connexion of the process with the activity of micro-organisms accomplishing the following functions in this process.

1. Micro-organisms decompose the initial organic residues to simpler compounds, some of which subsequently serve as structural elements for the formation of humic substances (e.g., aromatic compounds of polyphenol type from tannic substances and lignin).

2. Products of metabolism and products of resynthesis of bacterial plasma may serve as constituents for synthesis of humic substances.

3. The synthesis occurs under conditions of biocatalysis effected by oxidizing enzymes of the phenoloxidase type.

We shall now discuss the state of affairs which arises from contemporary theories on the biochemistry of the process and which is related to the problem of plant nutrition.

A close connexion between the process of humus formation and the activity of micro-organisms, the participation of the products of microbial metabolism in the formation of the particles of humic substances determine certain physiological functions of humic substances, but little attention has been paid to the study of these functions.

There is no doubt about the great importance of soil organic matter as the main reserve of plant nutrients (chiefly of nitrogen and phosphorus) and as a source of carbon dioxide for the carbon nutrition of plants.

It has been established that the plant utilizes firstly the CO_2 of the air layers near the ground. Here the soil is the main supplier of CO_2 , which is formed during root respiration and during the activity of (autotrophic and) mainly heterotrophic micro-

organisms utilizing soil organic matter as a source of energy.

Besides being a reserve of nutrients organic matter also plays an indirect role in mobilizing a series of elements from the mineral part of the soil. Solution of phosphates, CaCO_3 , MgCO_3 and also decomposition of rocks and minerals (kaolin, feldspar, garnet) with the formation of soluble forms of K_2O , Al_2O_3 , Fe_2O_3 , SiO_2 occur widely in nature. This is due to the action of products of microbial activity, e.g. CO_2 , organic acids (butyric, lactic, acetic, glucovanillic, oxalic and others) and also to that of bacterial mucus on the mineral part of the soil.

However, it would be a mistake to limit the importance of soil organic matter for plant nutrition to the above-mentioned aspects. More and more facts indicate the unique participation of organic matter in plant nutrition.

Here we shall limit ourselves to giving references to some review publications of recent years which throw light on a series of extremely interesting aspects characterizing the physiological properties of soil organic matter. In numerous papers (Kononova, 1951; Krasil'nikov, 1952; Rakitin, 1953; Ovcharov, 1953) the effect of a series of organic substances (mainly of aromatic nature), representing the products of decomposition of plant and animal residues and also products of activity and metabolism of micro-organisms on plant growth is noted, the effect being similar to that of vitamins and growth-stimulating substances.

The new experimental data of Ratner, Kolosov, *et al* (1956) are worth mentioning. Assuming that the basis of plant nutrition is the utilization of inorganic compounds of nitrogen and phosphorus, the authors stress the possibility of formative and other effects on the plant of small amounts of various organic nitrogen and phosphorus compounds absorbed by the roots, these compounds being products of the biological cycle of these elements in the soil. It is worth mentioning that the aromatic amino acid, phenylalanine, showed the most distinct effect on the plant.

The phenomenon of the stimulating effect of humic substances on plant growth is also very interesting. In a series of earlier studies (see Kononova, 1951) and in more recent works (Khris-teva, 1955; Chaminade and Blanchet, 1953, 1956; Flaig and Saalbach, 1956; Saalbach, 1956) data are given indicating the physiological effect of humic substances on the plant, this effect being shown by increased penetrability of the cell membranes, by participation in metabolism, and also by stimulation of the plant enzyme systems. This stimulation is connected with the presence of quinone groups in the humic substances; entering the plants in a highly dispersed state, humic substances, thanks to the presence of these groups, are included in the oxidation-reduction phenomena accompanying plant respiration and increase its energy.

This hypothesis on the participation of humic acids in plant nutrition was demonstrated by Khristeva in a simple experiment (1951, 1953). When a sol of humic acid was injected into plant tissue the rate of oxygen absorption increased (using a Warburg apparatus). Khristeva assumes that, because of the presence of quinone groups, humic acids are acceptors of hydrogen and activate oxygen thereby.

A similar explanation of the mechanism of stimulation of plant respiration by humic substances is found in the works of some foreign investigators (Flaig and Otto, 1951; Flaig, 1954). The correctness of this hypothesis is confirmed by experiments with anthraquinones (Otto, 1952) and with thymohydroquinone (Flaig and Saalbach, 1955, 1956); these compounds had the same effect as humic acids, obviously due to the presence of quinone groups in both substances.

Data indicating the effect of humic substances on the colloidal-chemical state of the protoplasm of plant cells are also interesting. Earlier investigations of Prozovskaya (1936), indicating the increased penetrability of the protoplasm of sunflower leaves in the presence of humic acid and the concomitant increase in the entry of nitrogen as NH_4NO_3 into the leaves are confirmed by more recent investigations. Thus Chaminade and Blanchet (1953), in experiments with the epidermis of *Viola cornuta* flowers, have found that the presence of humic substances in a solution of mineral salts hastened the occurrence of deplasmolysis in the plant cells and favoured the penetration of mineral elements through the cell membrane. This statement was confirmed by sand and soil cultures in glasshouse experiments (ryegrass and rye), where application of small amounts of humic acid increased the uptake of mineral nutrients, particularly of nitrogen and phosphorus, and in a number of cases increased yields (1951, 1956).

It is obvious that the specific effect of organic substances on plants cannot be regarded as a fundamental type of plant nutrition, which, as is well known, is based on the assimilation of inorganic forms of nitrogen, phosphorus, potassium and other elements by the plant. However, the specific effect of numerous organic substances (decomposition products of plant and animal residues, products of microbial activity and humic substances themselves)

that is similar to that of vitamins and growth-stimulating substances produces a high biological potential in the zone of root growth and increases the total vigour of the plant, thus favouring the more intensive and economical utilization of soil nutrients and fertilizers, and ultimately increasing plant growth and yields.

Certain facts already indicate possible uses of the specific properties of humic substances, not only for research purposes.

Such phenomena as the greater effectiveness of organo-mineral mixtures compared with mineral fertilizers, and also the positive effect of the placement of small amounts of well rotted manure to trees, flowers and vegetables are connected to some extent with the specific effect of humic substances.

The production of special fertilizers is worth consideration. These include humic fertilizers obtainable from peats (Khristeva) which, when applied in small amounts to chernozems of the southern zone, particularly in irrigated areas, give a positive effect with several crops. It is possible that products of the oil industry (Gusseĭnov, 1955, 1956) could be used as a raw material for the preparation of specific organic fertilizers. Tests have been carried out using small amounts of synthetic substances of quinone structure (thymohydroquinone—Flaig and Saalbach) for increasing crop yields.

The general principles in the preparation and application of these fertilizers that follow from the above theoretical concepts appear to be: the presence in fertilizers of substances of an aromatic nature, these substances having the greatest effect on the plant, the possibility of the conversion of humic substances into a highly dispersed state, favouring penetration into the plant. The effect of specific fertilizers is shown when they are applied in small amounts, in immediate contact with the plant-root system.

The specific effect on plants of organic substances, particularly of humic substances, determined by the biochemistry of their transformation in soil indicates future problems in the study of soil humus. Further investigations should include both an explanation of the mechanisms of their effect on plants and the development of possible means of utilizing these properties for increasing crop yields.

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